

# A combination of sorafenib and radiotherapy reduces NF- $\kappa$ B activity and growth of hepatocellular carcinoma in an orthotopic mouse model

HUI-YEN CHUANG<sup>1\*</sup>, YEU-SHENG TYAN<sup>2\*</sup>, JENG-JONG HWANG<sup>2,3</sup>,  
KUANG-CHUNG SHIH<sup>4,5</sup> and WEI-CHAN LIN<sup>1,6,7</sup>

<sup>1</sup>Department of Biomedical Imaging and Radiological Sciences, National Yang Ming Chiao Tung University, Taipei 112; <sup>2</sup>Department of Medical Imaging, Chung Shan Medical University Hospital; <sup>3</sup>Department of Medical Imaging and Radiological Sciences, Chung Shan Medical University, Taichung 402; <sup>4</sup>Division of Endocrinology and Metabolism, Department of Medicine, Cheng-Hsin General Hospital; <sup>5</sup>Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei 112; <sup>6</sup>Department of Radiology, Cathay General Hospital; <sup>7</sup>School of Medicine, Fu-Jen Catholic University, New Taipei 106, Taiwan, R.O.C.

Received February 12, 2020; Accepted January 18, 2021

DOI: 10.3892/ol.2021.12598

**Abstract.** Hepatocellular carcinoma (HCC) is difficult to diagnose at an early stage, and its prognosis is generally poor. Sorafenib is the primary treatment for unresectable advanced HCC and targets multiple receptor tyrosine kinases. However, sorafenib only extends the average survival time by 3 months. This observation indicates that sorafenib may need to be combined with other treatments to further improve outcomes. We previously showed that combination of sorafenib with radiotherapy (RT) enhances tumor inhibition in subcutaneous HCC mouse models compared with monotherapy. The present study demonstrated that combining sorafenib and RT could suppress tumor growth in an orthotopic HCC model by regulating apoptosis and NF- $\kappa$ B-related pathways. Moreover, decreased numbers of visible liver tumors and a smaller percentage of spleen metastases were found in the combination group. A transient drop in body weight was initially observed after RT, but progressive recovery of body weight occurred. The current study showed that the combination of sorafenib and RT could be a safe strategy for HCC treatment.

## Introduction

Hepatocellular carcinoma (HCC) is a common cause of cancer-associated death in Taiwan. Generally, HCC is most prevalent in Asia (~75% of cases were reported in Asia in 2015) compared with the rest of the world (1); however, HCC has become a common disease worldwide and is currently the ninth leading cause of cancer-associated death in the United States (2). Unlike other cancer types, the prognosis of HCC remains poor, although new diagnosis and treatment strategies have been developed. Late diagnosis is the main contributor to poor prognosis, and it strongly impacts overall HCC outcomes. Due to late diagnosis, only 30% of patients with HCC are eligible for surgery, and the remaining patients are usually given other treatments, including sorafenib, embolization and radiotherapy (RT).

The Sorafenib HCC Assessment Randomized Protocol trial conducted in 2008 showed that overall survival was prolonged by only 3 months when using sorafenib as a single agent to treat advanced HCC (3). Hence, sorafenib has been combined with conventional treatments, such as radiofrequency ablation (RFA) (4) and transarterial chemoembolization (TACE) (5), to enhance treatment outcomes and reduce the incidence of sorafenib-induced side effects. However, combining sorafenib with radiotherapy (RT) is controversial because both treatments have potential toxicities, including skin reactions and thrombosis (6-9).

The liver is a radiosensitive organ (10), and HCC tumors are relatively resistant to RT (11). Thus, RT is not the primary treatment for HCC. Hsieh *et al* (6) was the first group to report that improved HCC tumor control was observed in patients after treatment with a combination of sorafenib and RT, but severe skin reactions occurred. Since then, improved treatment outcomes resulting from the combination of sorafenib and RT have also been reported by other groups. However, severe side effects, such as hand-foot syndrome and gastrointestinal bleeding, have also been observed in patients receiving the combination treatment (7,8). When a large portion of the

---

*Correspondence to:* Dr Hui-Yen Chuang, Department of Biomedical Imaging and Radiological Sciences, National Yang Ming Chiao Tung University, 155 Section 2 Li-Nong Street, Beitou, Taipei 112, Taiwan, R.O.C.  
E-mail: d49920006@ym.edu.tw

\*Contributed equally

**Key words:** sorafenib, radiotherapy, hepatocellular carcinoma, orthotopic mouse model

liver needs to be irradiated, the combination of sorafenib and RT is not recommended because of its severe toxicity (9). Radioembolization is a minimally invasive procedure that merges the advantages of embolization and RT for HCC treatment (12,13). Radioembolization mostly uses  $\beta$ -emitters, which have relatively short penetration ranges, and minimizes the volume of liver irradiated, therefore decreasing the likelihood of RT-induced toxicities (14,15). A combination of sorafenib and radioembolization has been tested in patients; however, conflicting results have been found. Improved outcomes were observed by Mahvash *et al* (16), but Rieke *et al* (17) reported that the combination treatment did not improve outcomes and suggested that a improved trial design would be needed.

Yu *et al* (18) proposed that sorafenib enhances outcomes by inhibiting MAPK, NF- $\kappa$ B and VEGF pathways, suggesting that sorafenib should be given after RT. Our group previously demonstrated that sorafenib suppresses the ERK/NF- $\kappa$ B pathway and ameliorates the therapeutic efficacy of RT in oral cancer (19) and HCC (20) in orthotopic and subcutaneous models, respectively. As aforementioned, the volume of liver irradiated is strongly associated with radiation-induced toxicity and might influence treatment outcomes. Though our previous study did not observe severe toxicity caused by the combination of sorafenib and RT in the subcutaneous HCC model, it is important to understand whether this combination strategy works in an orthotopic HCC model. Therefore, the present study aimed to evaluate the effectiveness and safety of the combination of sorafenib with RT in an orthotopic HCC model and to study the possible underlying mechanisms. These results may improve our understanding of whether the combination of sorafenib and RT would be feasible and safe for HCC treatment in human clinical settings.

## Materials and methods

**Cell lines.** The human HCC cell line Huh7/NF- $\kappa$ B-*tk-luc2/rfp* used in this study was established previously (21). Huh7/NF- $\kappa$ B-*tk-luc2/rfp* cells were maintained in DMEM supplemented with 10% FBS and 1% PS (both HyClone; Cyvita) supplemented with 500  $\mu$ g/ml of G418 to maintain *tk* and *luc2* expression.

**Orthotopic HCC/NF- $\kappa$ B-*tk-luc2/rfp* tumor-bearing model.** In total, 40 7- to 8-week old male nude mice (average weight, 25 g) were used to generate the orthotopic HCC mouse model. All the mice had free access to food and water during the whole experimental period with a 12/12 h light/dark cycle. Briefly, mice were anesthetized with 1.5-2% isoflurane, and a 1- to 1.5-cm incision was made below the ensiform process after the surgical area was cleaned with iodine-alcohol. In total, 50  $\mu$ l of  $5 \times 10^5$  Huh7/NF- $\kappa$ B-*tk-luc2/rfp* cells mixed with Matrigel (cat no. 354263; Corning, Inc.) was slowly injected into the top lobe of the liver that was pushed out through the incision. Then the wound was sutured, and the mice were monitored and sent back to their cages until they recovered from anesthesia. Humane endpoints were set as follows: 20% Body weight loss, loss of mobility and activity. However, no mice reached these humane endpoints during the study.

Bioluminescent imaging (BLI) was used to track *in vivo* NF- $\kappa$ B activity. BLI was conducted at Taiwan Mouse Clinic.

Body weights were also monitored to assess the general toxicity caused by treatments. The tumor-bearing mice were randomly divided into four groups (n=5/group): Control, sorafenib (10 mg/kg/per day, peroral), radiotherapy (single dose, 6 Gy) and combination treatment. The detailed experimental design is shown in Fig. 1. The animal experiment was performed twice. The mice were euthanized on day 29 using carbon dioxide (20% volume displacement per minute), and their deaths were verified by checking eye color and reflex action. Then cervical dislocation was performed to ensure mice would not recover from CO<sub>2</sub> inhalation. All the animal experiments and procedures were approved by The Institutional Animal Care and Use Committee of National Yang-Ming University (Taipei, Taiwan; approval no. 1001238).

**BLI.** BLI was used to evaluate *in vivo* NF- $\kappa$ B activity on the designated days (days-6, 1, 8, 15, and 22). Briefly, mice were anesthetized with 1.5-2% isoflurane and injected with 150 mg/kg D-luciferin intraperitoneally. After 10 min, images were acquired over 5 min, and the photons emitted from tumors were detected using an *in vivo* Imaging system (Xenogen IVIS 50; Caliper Life Sciences). The images were analyzed using Living Imaging software 4.3.1 (PerkinElmer, Inc.). BLI signals emitted from the tumors were quantified and plotted against the days after the first treatment (three mice showing high BLI signals on day -6 were excluded).

**Electrophoretic mobility shift assay (EMSA).** Mice were sacrificed on day 29, and cytosolic and nuclear proteins were extracted from the tumors using a Nuclear Extraction kit (EMD Millipore). A LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc.) was utilized to assess NF- $\kappa$ B/DNA binding activity. The procedures were conducted according to the manufacturer's instructions. Nuclear proteins isolated from tumors were mixed with biotinylated DNA probes at room temperature for 20 min. The protein (20  $\mu$ g)/DNA probe mixtures were separated on a 5% polyacrylamide gel and transferred to nylon membranes. UV cross-linking was performed for permanent DNA fixation. ECL substrate provided with the EMSA kit was added to enhance signals after streptavidin-horseradish peroxidase incubation for 5 min at room temperature, and the signals were detected using X-ray film (Fujifilm Corporation). ImageJ 1.52 a (National Institutes of Health) was used for signal quantification. The following DNA sequences were synthesized for EMSA analysis: AGT TGAGGGGACTTTCCAGGC (Sense) and GCCTGGGAA AGTCCCCTCAACT (antisense) (20).

**Western blotting.** For *in vitro* analysis, cells treated with 6 Gy X-ray, 10  $\mu$ M sorafenib or combination treatment were harvested and lysed with NP-40 lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 1% NP-40). For *ex vivo* examination, mice were sacrificed on day 29. Proteins were extracted from tumors using tissue protein extraction reagent (Thermo Fisher Scientific, Inc.) to evaluate protein expression changes caused by the treatments. Protein concentrations were determined using a Bradford assay. In total, 30  $\mu$ g cell or tissue lysates were separated using 8-15% SDS-PAGE, transferred to PVDF membranes, and incubated with specific primary antibodies overnight at 4°C after 1-h blocking with 5% non-fat

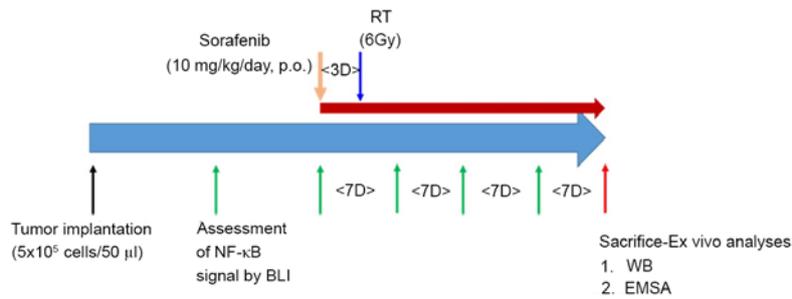


Figure 1. *In vivo* experimental design. Mice were injected with  $5 \times 10^5$  Huh7/NF- $\kappa$ B-*tk-luc2/rfp* cells intrahepatically to generate orthotopic hepatocellular carcinoma tumors. Mice were randomly divided into four groups according to BLI signals. RT group received 6 Gy of radiation 6 days after grouping, and both the sorafenib and combination treatment groups received their first sorafenib dose 3 days before irradiation. *In vivo* NF- $\kappa$ B activity was monitored by BLI once a week for 5 weeks, and the mice were sacrificed on day 29. Tumors were removed and analyzed using WB and EMSA. BLI, bioluminescent imaging; RT, radiotherapy; WB, western blotting; EMSA, electrophoretic mobility shift assay.

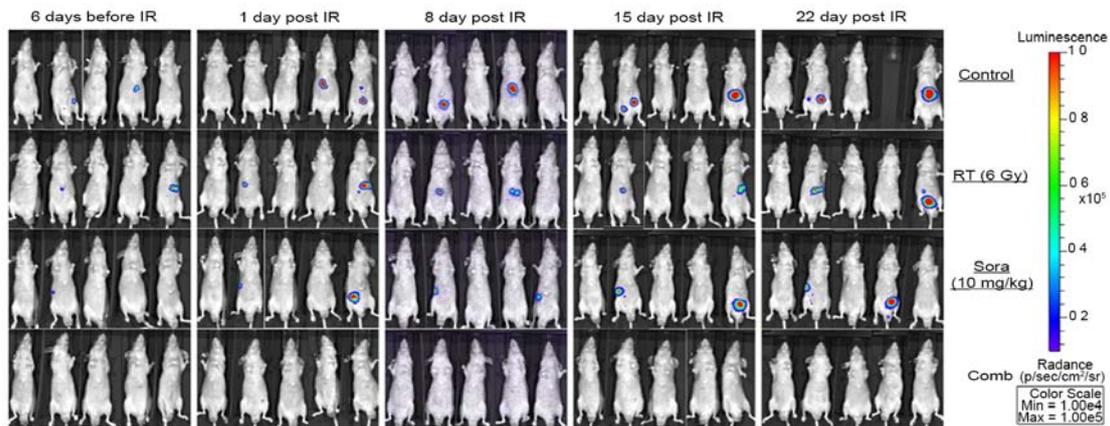


Figure 2. BLI used to monitor changes in NF- $\kappa$ B activity in orthotopic hepatocellular carcinoma tumor-bearing mice. *In vivo* NF- $\kappa$ B activity was tracked by BLI as Huh7 cells were stably transfected with the NF- $\kappa$ B-*tk-luc2/rfp* construct. Mice were randomly divided into four groups. All images are presented with the same scale bar to observe signal changes after treatment over time. BLI, bioluminescent imaging; IR, irradiation; RT, radiotherapy; Sora, sorafenib; Comb, combination.

milk at room temperature. Primary antibodies used in this study included anti-matrix metalloproteinase (MMP)-9 (cat. no. #13667), anti-cyclooxygenase (COX)-2 (cat. no. #12282), anti-cyclin D1 (cat. no. #55506), anti-cellular FLICE-like inhibitory protein (c-FLIP; cat. no. #8510), anti-caspase-8 (cat. no. #4790), anti-cleaved caspase-3 (cat. no. #9664), anti-Bcl-2 (cat. no. #4223), anti-Bcl-xL (cat. no. #2764), anti-Mcl-1 (cat. no. #5453), anti-Bcl-2-like protein 11 (Bim; cat. no. #2933), anti-Bak (cat. no. #12105), anti-BH3-interacting domain death agonist (BID; cat. no. #2002) and anti- $\beta$ -actin (cat. no. #3700). All primary antibodies were purchased from Cell Signaling Technology, Inc., and diluted to 1:1,000 in 1X TBST (TBS containing 0.1% Tween-20) before incubation with membranes, except for anti- $\beta$ -actin that was diluted to 1:5,000. Membranes were washed with 1X TBST and incubated with anti-rabbit and anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. nos. 5220-0337 and 5220-0339, respectively) with 1:10,000 dilution (SeraCare) at room temperature for 1 h. Finally, the proteins of interest were detected using an Enhanced Chemiluminescent system (MilliporeSigma) and the LAS-4000 imaging system (Fujifilm, Corporation). The band intensities were quantified using ImageJ, and  $\beta$ -actin served as an internal control.

**Statistics.** All the *in vitro* experiments were repeated three times, and the animal studies were repeated twice. GraphPad Prism 8 (GraphPad Software) was used to generate plots and perform statistical analyses. All the results are presented as mean  $\pm$  standard error or the mean. One-way ANOVA and Tukey's post hoc tests were performed to compare difference between groups for western blotting. Two-way ANOVAs and Tukey's post hoc tests were performed to compare differences between groups for the animal studies, and the two variables were treatments and time.

## Results

*In vivo* BLI shows that combination of sorafenib and RT results in the most significant NF- $\kappa$ B suppression in orthotopic HCC tumors. The NF- $\kappa$ B/*tk-luc2* construct contains an NF- $\kappa$ B-responsive element to drive the downstream reporter genes, *tk* and *luc2*, which allows determination of NF- $\kappa$ B activity using imaging. We previously established a stable clone named Huh7/ NF- $\kappa$ B/*tk-luc2* (21). Using these cells, the present study monitored NF- $\kappa$ B activity in HCC tumors using *in vivo* BLI on the same cohorts of mice over time. Except for three, the mice had minimal BLI signals on day 6 (Fig. 2). All the images shown in Fig. 2 have the same scale bar, which helps

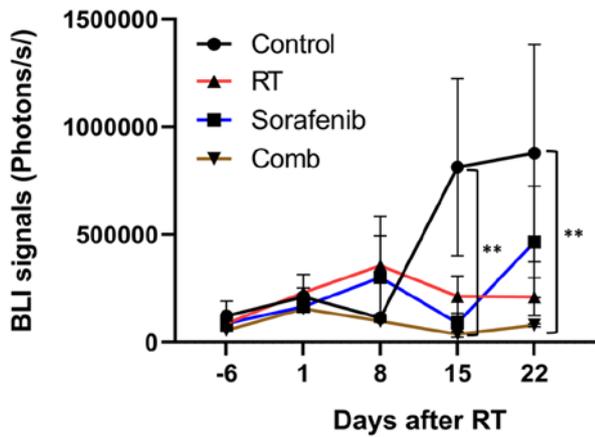


Figure 3. Comb treatment suppresses *in vivo* NF- $\kappa$ B activity in orthotopic hepatocellular carcinoma tumors. \*\*\* $P < 0.01$  compared with control group. RT, radiotherapy; BLI, bioluminescent imaging; Comb, combination.

observe longitudinal signal changes. Fig. 2 shows that the BLI signals increased over time in the control, RT and sorafenib groups. Notably, the BLI signal did not change significantly in the combination group.

Fig. 3 shows quantified BLI results of all groups. On day 15, significant differences were first detected in both the sorafenib and combination groups compared with the control group ( $P < 0.01$ ). Moreover, a significant difference between the control and combination groups ( $P < 0.01$ ) was still observed on day 22.

It is worth noting that the average BLI signal of RT group was comparable to that of the sorafenib group until day 15, but an increased signal was observed in the RT group on day 22. The trends in BLI signal changes were similar between the sorafenib and combination groups until day 22 after RT, and the average BLI signal was slightly higher in the sorafenib group compared with in the combination group.

*Combination of sorafenib and RT suppresses NF- $\kappa$ B/DNA binding ability and represses NF- $\kappa$ B regulated proteins in orthotopic HCC tumors.* As shown in Fig. 4, RT did not decrease NF- $\kappa$ B/DNA binding ability, which was suppressed by sorafenib. Also, combination treatment reduced NF- $\kappa$ B/DNA binding ability in orthotopic HCC tumors. These results are consistent with the aforementioned *in vivo* BLI observations.

The EMSA results indicated that combination treatment strongly reduced NF- $\kappa$ B/DNA binding ability in orthotopic HCC tumors. NF- $\kappa$ B is a signaling transduction hub and regulates several pathways associated with metastasis, inflammatory tumor microenvironments, proliferation and anti-apoptosis (22). Therefore, the expression of the following proteins was examined: MMP-9, COX-2, Cyclin D1 and c-FLIP. Fig. 5 shows that MMP-9, COX-2 and cyclin D1 were suppressed by RT, sorafenib and combination treatment. Notably, c-FLIP expression was decreased by sorafenib but elevated after RT, and the combination treatment resulted in slight c-FLIP suppression compared with the control group. Lastly, the levels of caspase-8 and cleaved caspase-3 were examined. RT failed to increase caspase-8 and cleaved caspase-3 levels, unlike sorafenib and combination treatment significantly increased cleaved caspase-3 expression

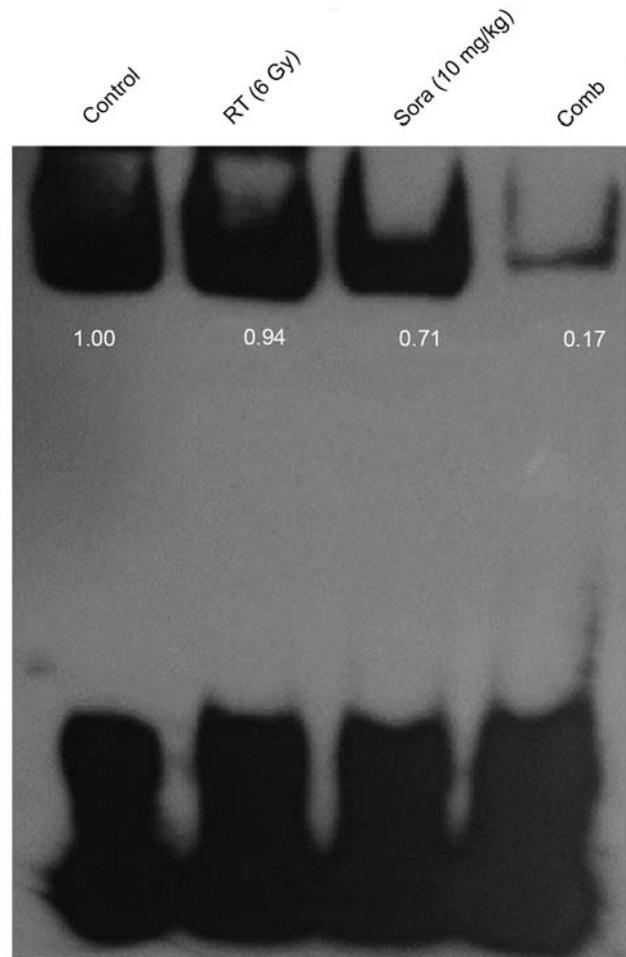


Figure 4. Comb treatment strongly represses NF- $\kappa$ B/DNA binding activity in orthotopic HCC tumors. Nuclear proteins were isolated from orthotopic hepatocellular carcinoma tumors and analyzed using an electrophoretic mobility shift assay to examine NF- $\kappa$ B/DNA binding activity. Comb, combination; Sora, sorafenib; RT, radiotherapy.

in orthotopic HCC tumors ( $P < 0.01$  compared with control;  $P < 0.05$ ,  $P < 0.01$  compared with RT;  $P < 0.01$  compared with sorafenib) (Fig. 5).

Cells treated with 6 Gy RT, 10  $\mu$ M sorafenib or combination treatment were analyzed using western blotting to determine how these treatments influenced Bcl-2 family proteins and subsequent signaling pathways. As shown in Fig. 6, Bcl-2 and pro-survival Bcl-xL and Mcl-1 were slightly decreased in all treatment groups. However, sorafenib and combination treatment significantly elevated the expression of pro-apoptotic Bim, BAK and BID compared with control group ( $P < 0.05$  and  $P < 0.01$  compared with control;  $P < 0.01$  compared with RT;  $P < 0.05$  compared with sorafenib).

*Combination treatment not only suppresses NF- $\kappa$ B/DNA binding activity but also inhibits the growth of orthotopic HCC tumors.* Fig. 7 shows representative tumors removed from control mice and those that received different treatments. The images show that all treatments shrank the orthotopic HCC tumors, and the combination treatment resulted in the most notable tumor inhibition. The largest tumor diameters found in each group were also compared (Table I). Visible HCC tumors were still found in more than half of the mice

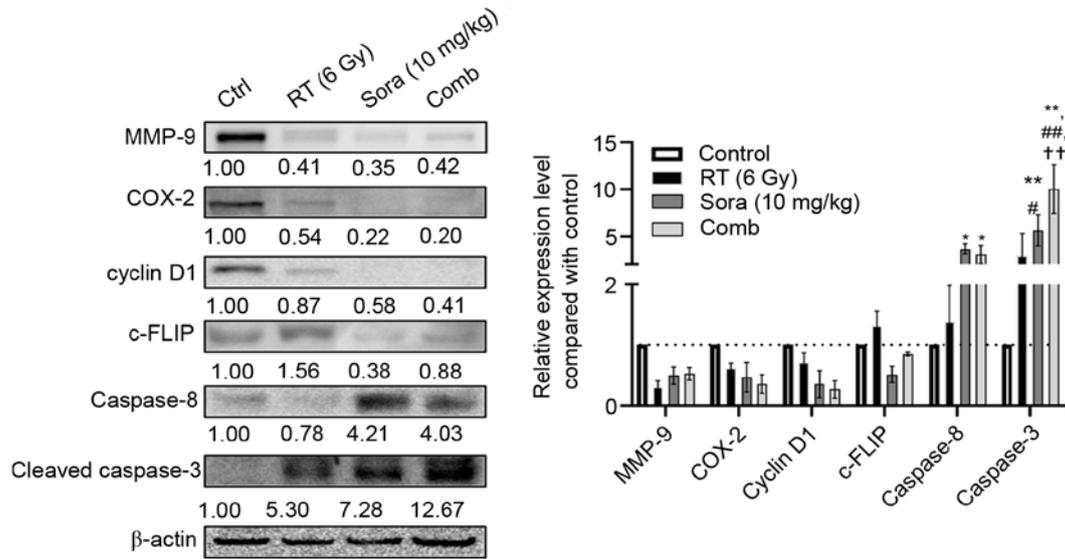


Figure 5. Western blotting demonstrates that comb treatment regulates the expression of NF-κB-regulated and apoptosis-related proteins. \*P<0.05 and \*\*P<0.01 compared with control group, #P<0.05 and ##P<0.01 compared with RT group and ††P<0.01 compared with Sora group. Ctrl, control; Sora, sorafenib; RT, radiotherapy; Comb, combination; MMP, matrix metalloproteinase; c-FLIP, cellular FLICE-like inhibitory protein; COX, cyclooxygenase.

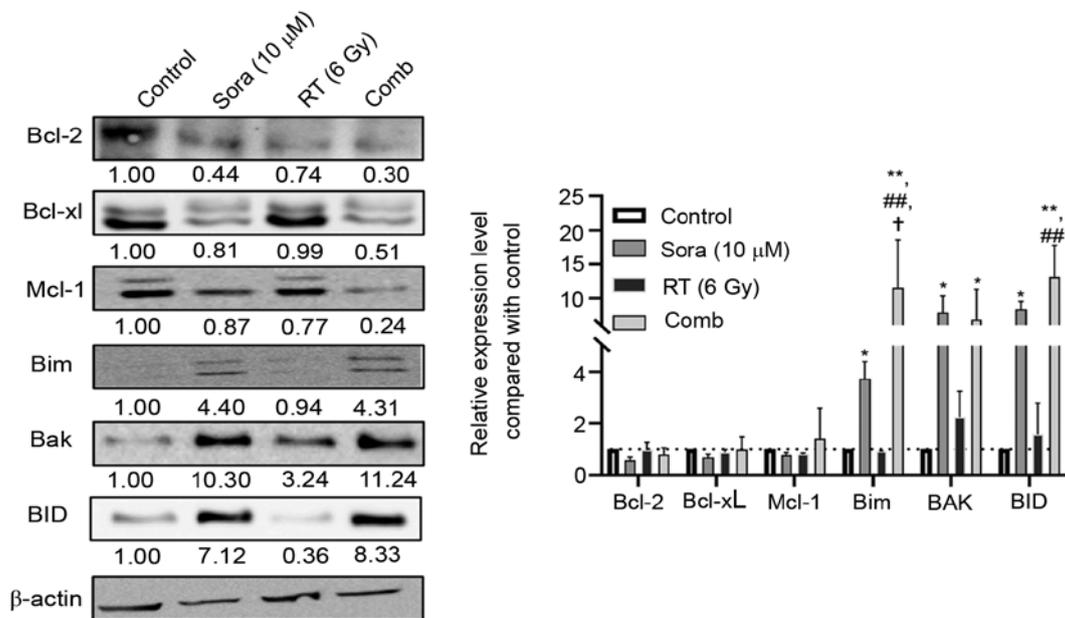


Figure 6. Sorafenib and the combination treatment contribute to cell death by enhancing the expression of Bcl-2-related pro-apoptotic proteins. \*P<0.05 and \*\*P<0.01 compared with control group, #P<0.01 compared with RT group and †P<0.05 compared with Sora group. Sora, sorafenib; RT, radiotherapy; Comb, combination; Bim, Bcl-2-like protein 11; BID, BH3-interacting domain death agonist.

after treatment; however, the largest tumor diameters detected in the treatment groups were smaller compared with those in the control group. Moreover, there were fewer total tumors found in the combination group compared with in the other three groups. All 10 mice in the control group had visible liver tumors (establishment rate=100%). Both sorafenib and combination treatment reduced the numbers of mice with visible liver tumors to six out of nine (establishment rate=67%). Fig. 8 shows transient but significant decreases in body weight were detected in the combination group on day 2 post RT. The body weight decrease was within ±20% of the mean body weight and recovered at later time points.

**Discussion**

HCC is mainly treated by surgical removal; however, only small numbers of patients can have surgery because of tumor location and numbers (23). Patients with unresectable HCC may undergo RFA, TACE, chemotherapy and RT, but none of these treatments are efficient for advanced HCC (24,25). Sorafenib is a targeted therapy for advanced HCC, and it blocks signaling pathways initiated by different receptor tyrosine kinases (RTKs), such as VEGF and platelet-derived growth factor (26). RT is not the first-line treatment for HCC because HCC is relatively resistant to RT compared with other cancer

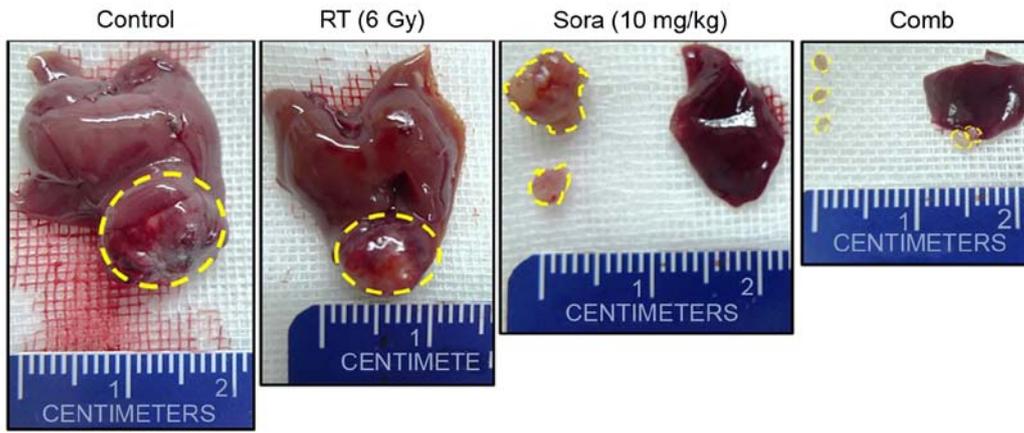


Figure 7. Representative pictures of the tumors from each group. Yellow dotted circles represent the hepatocellular carcinoma tumors. Sora, sorafenib; RT, radiotherapy; Comb, combination.

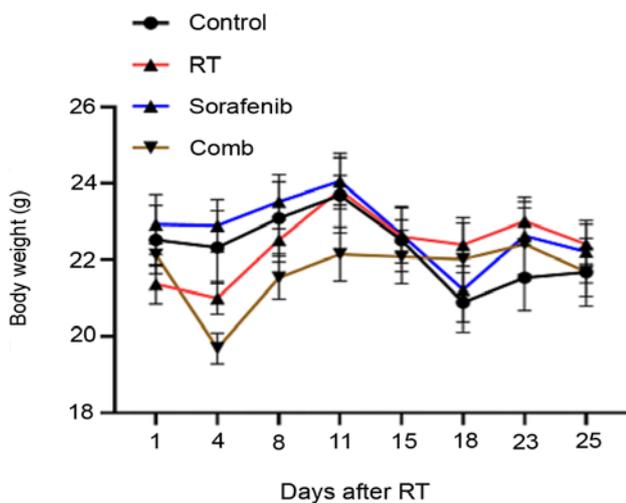


Figure 8. Combination treatment causes transient decreases in body weight in orthotopic hepatocellular carcinoma-bearing mice. RT, radiotherapy; Comb, combination.

types, such as lymphoma and head and neck cancer (27). HCC cells that survive RT exhibit more aggressive behaviors, including proliferation, anti-apoptosis and metastasis (28). Proteins associated with these aggressive behaviors, such as cyclin D1, Bcl-2 and MMP-9, are regulated by a key transcriptional factor, NF- $\kappa$ B. NF- $\kappa$ B activity modulates the balance between RT-induced apoptosis and radioresistance that influences the efficacy of RT in certain types of cancer, including oropharyngeal cancer, HCC and lung cancer (29-32).

Our previous study showed that pretreatment with sorafenib combined with RT led to improved tumor inhibition in subcutaneous HCC tumor-bearing mice through inhibition of NF- $\kappa$ B activity (20). The present study aimed to understand whether this combination treatment is also feasible and safe for application in an orthotopic HCC model because the liver is a relatively radiosensitive organ (6). Huh7/NF- $\kappa$ B-*tk-luc2/rfp* cells were used to generate an orthotopic HCC model. As the NF- $\kappa$ B-responsive element controls both reporter genes, *tk* and *luc2*, molecular imaging could be used to monitor *in vivo* NF- $\kappa$ B activity longitudinally with the same cohorts of mice (21).

*In vivo* BLI revealed changes in NF- $\kappa$ B activity over time. BLI signals were similar among all groups until day 15. Unlike the results obtained from previous subcutaneous HCC models (20), RT and sorafenib slightly increased NF- $\kappa$ B activity in orthotopic HCC tumors over time. Notably, NF- $\kappa$ B activity decreased over time in the combination group.

Then, nuclear proteins were extracted and EMSA was used to determine NF- $\kappa$ B activity; the results were consistent with the BLI findings. The combination treatment suppressed NF- $\kappa$ B/DNA binding activity, which was also repressed in the sorafenib and RT groups. Similar patterns, NF- $\kappa$ B-driven BLI signals and EMSA results have been observed in our previous studies (20,21). It has been reported that the NF- $\kappa$ B-driven reporter assay is more sensitive compared with EMSA because the use of a specific promoter/responsive element-driven reporter system could further enhance signals via both transcription and translation (33,34). Additionally, luciferase is not expressed by mammalian cells; thus, BLI should have minimal background signals and should detect small differences between groups (35).

NF- $\kappa$ B is a signaling hub controlling multiple proteins such as VEGF and XIAP and promoting tumor progression (22). The present study extracted proteins from tumors and used western blotting to examine the expression of MMP-9, cyclin D1, and COX-2, which are associated with invasiveness (36), proliferation (36) and inflammation (37). Although the expression of these proteins was found to be decreased in all treatment groups, they were reduced very little in the RT group compared with the other groups. Our previous studies (20,21) indicated that sorafenib slows HCC growth through ERK/NF- $\kappa$ B inhibition. Therefore, NF- $\kappa$ B activity was also examined, and the result was consistent with those for the three NF- $\kappa$ B downstream proteins aforementioned. Sorafenib and combination treatment markedly suppressed cyclin D1 expression in tumors.

Both anti-proliferation and increased apoptosis can lead to tumor suppression (38). Therefore, apoptosis-related proteins, including c-FLIP, caspase-8 and cleaved caspase-3, were further examined. Cleaved caspase-3 expression was increased in all treatment groups. It is worth noting that RT caused increased c-FLIP and decreased caspase-8 expression compared with the control group; however, c-FLIP was decreased and caspase-8 was

Table I. Rates of orthotopic HCC establishment, the total tumor numbers and the largest tumor diameter detected in each group.

Treatment group	HCC establishing rate, % <sup>a</sup>	Largest tumor diameter, mm <sup>b</sup>	Total tumor number <sup>c</sup>
Control	100	24	33
RT	90	10	16
Sorafenib	70	10	18
Combination	67	7	16

<sup>a</sup>Mice with visible orthotopic liver tumors at the last time point in ten mice from two separate experiments. <sup>b</sup>Largest largest tumor diameters found in all the tumors in each group. <sup>c</sup>Total numbers of tumors counted at the last time point in each group. HCC, hepatocellular carcinoma; RT, radiotherapy.

increased in the sorafenib and combination groups. The apoptotic pathway can be further divided into extrinsic and intrinsic pathways, and caspase-8 is present in both pathways. c-FLIP is known as a master anti-apoptosis regulator (39) and prevents activation of caspase-8 and its downstream caspase cascades. Stagni *et al* (40) proposed that ATM activation may modulate c-FLIP expression in lymphoid cells. The ATM serine/threonine kinase is activated by DNA damage resulting from chemotherapy or ionizing radiation, then initiates DNA repair or apoptotic processes. Ivanov *et al* (41) showed that pretreatment with an ATM inhibitor, KU-55933, decreased radiation-induced c-FLIP, p53 and NF- $\kappa$ B activation in melanoma cells. c-FLIP-silencing can also enhance TNF-related apoptosis-inducing ligand (TRAIL)-mediated cell killing by restoring apoptosis in cervical, ovarian and breast cancer cells (42,43).

It is known that RT-induced apoptosis occurs mainly through the intrinsic pathway (44), and this could partially explain the present western blotting results. RT increased cleaved caspase-3 and c-FLIP expression, and reduced caspase-8 expression compared with the control group. These results implied that the development of radioresistance is not only caused by NF- $\kappa$ B but also c-FLIP in orthotopic HCC tumors. McLaughlin *et al* (45) reported that c-FLIP expression negatively modulates radiosensitivity in non-small cell lung cancer by overexpressing and silencing c-FLIP. c-FLIP is also one of the proteins downstream of NF- $\kappa$ B (46).

NF- $\kappa$ B and c-FLIP influence Bcl-2 family protein expression through different mechanisms. NF- $\kappa$ B transcriptionally promotes Bcl-2 expression (47), and c-FLIP prevents caspase-8-mediated BID cleavage and intrinsic apoptosis (48). Bcl-2 family proteins can trigger both pro-apoptotic and pro-survival pathways (49). All treatments slightly suppressed the expression of Bcl-2 and pro-survival Bcl-2 family proteins including Bcl-xL and Mcl-1 in the present study. In contrast, Bcl-2-related pro-apoptotic Bim, Bak and BID were markedly increased after sorafenib and combination treatment. These results indicated that sorafenib and combination treatment promoted cell death mainly by enhancing pro-apoptotic signaling in HCC cells. Several studies have shown that the combination of sorafenib with Mcl-1 (50,51), Bcl-2 (52) or

Bcl-xL (53) inhibitors synergistically enhance its ability to kill different types of cancer cells.

Bidirectional regulation between NF- $\kappa$ B and c-FLIP has been reported (39). c-FLIP upregulates NF- $\kappa$ B expression (54) and enhances its nuclear translocation (55). However, the present EMSA results showed that RT did not change NF- $\kappa$ B/DNA binding activity, even when c-FLIP expression was increased in orthotopic HCC tumors. These results suggested that other transcription factors are involved. Accumulating evidence demonstrates that STAT3 inhibition could suppress STAT3 and reverse TRAIL resistance in multiple cancer types, such as lung cancer, renal carcinoma and HCC (56,57). Sorafenib inhibits tumor growth and metastasis by targeting RTKs and blocking the STAT3 pathway in HCC (58,59). Additionally, sorafenib has been shown to enhance radiation-induced apoptosis (60) and reverses TRAIL resistance (61) in HCC by targeting STAT3. There remain some interesting avenues for future research, for example the changes and interactions between STAT3 and NF- $\kappa$ B after sorafenib combined with radiotherapy, the roles of immune cells in sorafenib combined with radiotherapy and the possibilities of combining sorafenib, radiotherapy and immunotherapy in HCC. Although the changes in NF- $\kappa$ B activity in tumors were evaluated using EMSA, the NF- $\kappa$ B protein level was not determined in the current study. The changes in NF- $\kappa$ B-regulated Bcl-2 family proteins by treatments were studied with Huh7 cells rather than HCC tumors. The lack of *in vivo* experiments to confirm these findings is also a limitation of the present study.

Decreased cyclin D1 and elevated cleaved caspase-3 levels from tumors harvested in the present study were detected using western blotting, suggesting tumor reduction may have resulted from impaired cell proliferation and enhanced apoptosis, respectively. Moreover, all treatment groups showed smaller and fewer tumors compared with the control group. Again, the smaller tumor sizes and fewer metastatic lesions are consistent with the results obtained by western blotting. All treatments decreased cyclin D1 and MMP-9 expression and increased cleaved caspase-3 expression in orthotopic HCC tumors.

As the liver is a radiosensitive organ, potential toxicity limits RT applications in HCC treatment. Although changes in body weight were not observed in the subcutaneous HCC models (18), transient but significant decreases in body weight were detected in the combination group in the current study. However, the body weight decrease was within  $\pm 20\%$  of the mean body weight and recovered at later time points. The reduction in body weight was due to RT-induced toxicity rather than cachexia. Although only the liver was exposed to RT during irradiation, intestinal damage may have occurred contributing to weight loss. It was speculated that the toxicity resulting from combination treatment would be acceptable. The transient toxicity could be further decreased if the irradiation dose could be delivered more precisely and exclusively to tumors. Particle therapies like proton therapy and carbon ion therapy could be ideal candidates. Both protons and carbon ions have narrow Bragg peak, which means that irradiation doses can be delivered at specific depths and reduce the doses received by surrounding normal tissues (62,63).

To the best of our knowledge, the present study is the first showing that the combination of sorafenib and RT could suppress tumor growth using an orthotopic HCC model.

In summary, pretreatment with sorafenib plus RT led to the best tumor inhibition with acceptable general toxicity. The sorafenib and RT combination acts through NF- $\kappa$ B inhibition and likely STAT3 suppression as well. Moreover, it would be worth investigating how the combination treatment modulates tumor metastasis and influences the tumor microenvironment in the future as both NF- $\kappa$ B and STAT3 are critical signaling hubs that regulate cancer progression (64-66).

### Acknowledgments

Not applicable.

### Funding

The study was supported by grants from The Ministry of Science and Technology (grant no. NSC101-2314-B-010-045-MY3) and The Chung Shan Medical University Hospital (grant no. CSH-2017-C008).

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

HYC carried out experimental work, data analysis, manuscript preparation and editing. YST assisted with data analysis, manuscript preparation and editing. KCS assisted with western blotting experiments. WCL assisted with experimental design, data analysis and manuscript editing. JJH designed and supervised the experiments, edited the manuscript and acquired funding. HYC and JJH confirmed the authenticity of all raw data. All authors have read and approved the final manuscript.

### Ethics approval and consent to participate

All the animal experiments and procedures were approved by The Institutional Animal Care and Use Committee of National Yang-Ming University (Taipei, Taiwan; approval no. 1001238).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

- McGlynn KA, Petrick JL and London WT: Global epidemiology of hepatocellular carcinoma: An emphasis on demographic and regional variability. *Clin Liver Dis* 19: 223-238, 2015.
- Golabi P, Fazel S, Otgonsuren M, Sayiner M, Locklear CT and Younossi ZM: Mortality assessment of patients with hepatocellular carcinoma according to underlying disease and treatment modalities. *Medicine (Baltimore)* 96: e5904-e5904, 2017.
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, *et al*: Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 359: 378-390, 2008.
- Chen L, Sun J and Yang X: Radiofrequency ablation-combined multimodal therapies for hepatocellular carcinoma: Current status. *Cancer Lett* 370: 78-84, 2016.
- Chao Y, Chung YH, Han G, Yoon JH, Yang J, Wang J, Shao GL, Kim BI and Lee TY: The combination of transcatheter arterial chemoembolization and sorafenib is well tolerated and effective in Asian patients with hepatocellular carcinoma: Final results of the START trial. *Int J Cancer* 136: 1458-1467, 2015.
- Hsieh CH, Jeng KS, Lin CC, Chen CK, Liu CY, Lin CP, Tai HC, Wang CH, Shueng PW and Chen YJ: Combination of sorafenib and intensity modulated radiotherapy for unresectable hepatocellular carcinoma. *Clin Drug Investig* 29: 65-71, 2009.
- Cha J, Seong J, Lee IJ, Kim JW and Han KH: Feasibility of Sorafenib combined with local radiotherapy in advanced hepatocellular carcinoma. *Yonsei Med J* 54: 1178-1185, 2013.
- Chen SW, Lin LC, Kuo YC, Liang JA, Kuo CC and Chiou JF: Phase 2 study of combined sorafenib and radiation therapy in patients with advanced hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 88: 1041-1047, 2014.
- Brade AM, Ng S, Brierley J, Kim J, Dinniwell R, Ringash J, Wong RR, Cho C, Knox J and Dawson LA: Phase 1 trial of sorafenib and stereotactic body radiation therapy for hepatocellular carcinoma. *Int J Radiat Oncol Biol Phys* 94: 580-587, 2016.
- Stryker JA: Science to practice: Why is the liver a radiosensitive organ? *Radiology* 242: 1-2, 2007
- Kalogeridi MA, Zygogianni A, Kyrgias G, Kouvaris J, Chatziioannou S, Kelekis N and Kouloulis V: Role of radiotherapy in the management of hepatocellular carcinoma: A systematic review. *World J Hepatol* 7: 101-112, 2015.
- Ricke J, Bulla K, Kolligs F, Peck-Radosavljevic M, Reimer P, Sangro B, Schott E, Schütte K, Verslype C, Walecki J, *et al*: Safety and toxicity of radioembolization plus Sorafenib in advanced hepatocellular carcinoma: Analysis of the European multicentre trial SORAMIC. *Liver Int* 35: 620-626, 2015.
- Riaz A, Gabr A, Abouchaleh N, Ali R, Al Asadi A, Mora R, Kulik L, Desai K, Thornburg B, Mouli S, *et al*: Radioembolization for hepatocellular carcinoma: Statistical confirmation of improved survival in responders by landmark analyses. *Hepatology* 67: 873-883, 2018.
- Guha C and Kavanagh BD: Hepatic radiation toxicity: Avoidance and amelioration. *Semin Radiat Oncol* 21: 256-263, 2011.
- Seidensticker R, Seidensticker M, Damm R, Mohnike K, Schütte K, Malfertheiner P, Van Buskirk M, Pech M, Amthauer H and Ricke J: Hepatic toxicity after radioembolization of the liver using (90)Y-microspheres: Sequential lobar versus whole liver approach. *Cardiovasc Intervent Radiol* 35: 1109-1118, 2012.
- Mahvash A, Murthy R, Odisio BC, Raghav KP, Girard L, Cheung S, Nguyen V, Ensor J, Gadani S, Elsayes KM, *et al*: Yttrium-90 resin microspheres as an adjunct to sorafenib in patients with unresectable hepatocellular carcinoma. *J Hepatocell Carcinoma* 3: 1-7, 2016.
- Ricke J, Klumpen HJ, Amthauer H, Bargellini I, Bartenstein P, de Toni EN, Gasbarrini A, Pech M, Peck-Radosavljevic M, Popovič P, *et al*: Impact of combined selective internal radiation therapy and sorafenib on survival in advanced hepatocellular carcinoma. *J Hepatol* 71: 1164-1174, 2019.
- Yu W, Gu K, Yu Z, Yuan D, He M, Ma N, Lai S, Zhao J, Ren Z, Zhang X, *et al*: Sorafenib potentiates irradiation effect in hepatocellular carcinoma in vitro and in vivo. *Cancer Lett* 329: 109-117, 2013.
- Hsu FT, Chang B, Chen JC, Chiang IT, Liu YC, Kwang WK and Hwang JJ: Synergistic effect of sorafenib and radiation on human oral carcinoma in vivo. *Sci Rep* 5: 15391, 2015.
- Chen JCH, Chuang HY, Hsu FT, Chen YC, Chien YC and Hwang JJ: Sorafenib pretreatment enhances radiotherapy through targeting MEK/ERK/NF- $\kappa$ B pathway in human hepatocellular carcinoma-bearing mouse model. *Oncotarget* 7: 85450-85463, 2016.
- Wang WH, Chiang IT, Liu YC, Hsu FT, Chen HW, Chen CL, Lee YJ, Lin WJ and Hwang JJ: Simultaneous imaging of temporal changes of NF- $\kappa$ B activity and viable tumor cells in Huh7/NF- $\kappa$ B-tk-luc2/rfp tumor-bearing mice. *In Vivo* 27: 339-350, 2013.
- Hoessel B and Schmid JA: The complexity of NF- $\kappa$ B signaling in inflammation and cancer. *Mol Cancer* 12: 86, 2013.
- Zamora-Valdes D, Taner T and Nagorney DM: Surgical treatment of hepatocellular carcinoma. *Cancer Control* 24: 1073274817729258, 2017.
- Eggert T and Greten TF: Current standard and future perspectives in non-surgical therapy for hepatocellular carcinoma. *Digestion* 96: 1-4, 2017.

25. Huang A, Yang XR, Chung WY, Dennison AR and Zhou J: Targeted therapy for hepatocellular carcinoma. *Signal Transduct Target Ther* 5: 146, 2020.
26. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM and Lynch M: Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. *Mol Cancer Ther* 7: 3129-3140, 2008.
27. Hayabuchi N: Radiocurable tumors and non-radiocurable tumors. *Japan Med Assoc J* 47: 79-83, 2004.
28. Piao LS, Hur W, Kim TK, Hong SW, Kim SW, Choi JE, Sung PS, Song MJ, Lee BC, Hwang D and Yoon SK: CD133<sup>+</sup> liver cancer stem cells modulate radioresistance in human hepatocellular carcinoma. *Cancer Lett* 315: 129-137, 2012.
29. Li F and Sethi G: Targeting transcription factor NF-kappaB to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta Rev Cancer* 1805: 167-180, 2010.
30. Qiao Q, Sun C, Han C, Han N, Zhang M and Li G: Endoplasmic reticulum stress pathway PERK-eIF2 $\alpha$  confers radioresistance in oropharyngeal carcinoma by activating NF- $\kappa$ B. *Cancer Sci* 108: 1421-1431, 2017.
31. Ren K, Li Z, Li Y, Zhang W and Han X: Sulforaphane enhances radiosensitivity of hepatocellular carcinoma through suppression of the NF- $\kappa$ B pathway. *J Biochem Mol Toxicol* 31: e21917, 2017.
32. Ji K, Sun X, Liu Y, Du L, Wang Y, He N, Wang J, Xu C and Liu Q: Regulation of apoptosis and radiation sensitization in lung cancer cells via the Sirt1/NF- $\kappa$ B/smact pathway. *Cell Physiol Biochem* 48: 304-316, 2018.
33. Smartt HJ, Elder DJ, Hicks DJ, Williams NA and Paraskeva C: Increased NF-kappaB DNA binding but not transcriptional activity during apoptosis induced by the COX-2-selective inhibitor NS-398 in colorectal carcinoma cells. *Br J Cancer* 89: 1358-1365, 2003.
34. Vanden Bergh W, Dijsselbloem N, Vermeulen L, Ndlovu MN, Boone E and Haegeman G: Attenuation of mitogen- and stress-activated protein kinase-1-driven nuclear factor-kappaB gene expression by soy isoflavones does not require estrogenic activity. *Cancer Res* 66: 4852-4862, 2006.
35. Close DM, Xu T, Saylor GS and Ripp S: In vivo bioluminescent imaging (BLI): Noninvasive visualization and interrogation of biological processes in living animals. *Sensors (Basel)* 11: 180-206, 2011.
36. Kunnumakkara AB, Diagaradjane P, Anand P, Harikumar KB, Deorukhkar A, Gelovani J, Guha S, Krishnan S and Aggarwal BB: Curcumin sensitizes human colorectal cancer to capecitabine by modulation of cyclin D1, COX-2, MMP-9, VEGF and CXCR4 expression in an orthotopic mouse model. *Int J Cancer* 125: 2187-2197, 2009.
37. Bachmeier BE, Killian PH and Melchart D: The role of curcumin in prevention and management of metastatic disease. *Int J Mol Sci* 19: 1716, 2018.
38. Lai ZC, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, Ho LL and Li Y: Control of cell proliferation and apoptosis by mob as tumor suppressor. *Mats. Cell* 120: 675-685, 2005.
39. Safa AR: c-FLIP, a master anti-apoptotic regulator. *Exp Oncol* 34: 176-184, 2012.
40. Stagni V, di Bari MG, Cursi S, Condò I, Cencioni MT, Testi R, Lerenthal Y, Cundari E and Barilà D: ATM kinase activity modulates Fas sensitivity through the regulation of FLIP in lymphoid cells. *Blood* 111: 829-837, 2008.
41. Ivanov VN, Zhou H, Partridge MA and Hei TK: Inhibition of ataxia telangiectasia mutated kinase activity enhances TRAIL-mediated apoptosis in human melanoma cells. *Cancer Res* 69: 3510-3519, 2009.
42. Kim TE, Hong S, Song K, Park SH and Shin YK: Sensitization of glycoengineered interferon- $\beta$ 1a-resistant cancer cells by cFLIP inhibition for enhanced anti-cancer therapy. *Oncotarget* 8: 13957-13970, 2017.
43. Poondla N, Chandrasekaran AP, Heese K, Kim KS and Ramakrishna S: CRISPR-mediated upregulation of DR5 and downregulation of cFLIP synergistically sensitize HeLa cells to TRAIL-mediated apoptosis. *Biochem Biophys Res Commun* 512: 60-65, 2019.
44. Maier P, Hartmann L, Wenz F and Herskind C: Cellular pathways in response to ionizing radiation and their targetability for tumor radiosensitization. *Int J Mol Sci* 17: 102, 2016.
45. McLaughlin KA, Nemeth Z, Bradley CA, Humphreys L, Stasik I, Fenning C, Majkut J, Higgins C, Crawford N, Holohan C, *et al*: FLIP: A targetable mediator of resistance to radiation in non-small cell lung cancer. *Mol Cancer Ther* 15: 2432-2441, 2016.
46. Ranjan K and Pathak C: FADD regulates NF-kappaB activation and promotes ubiquitination of cFLIPL to induce apoptosis. *Sci Rep* 6: 22787, 2016.
47. Catz SD and Johnson JL: Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 20: 7342-7351, 2001.
48. Engels IH, Stepczynska A, Stroh C, Lauber K, Berg C, Schwenzler R, Wajant H, Jänicke RU, Porter AG, Belka C, *et al*: Caspase-8/FLICE functions as an executioner caspase in anti-cancer drug-induced apoptosis. *Oncogene* 19: 4563-4573, 2000.
49. Delbridge AR and Strasser A: The BCL-2 protein family, BH3-mimetics and cancer therapy. *Cell Death Differ* 22: 1071-1080, 2015.
50. Tong J, Wang P, Tan S, Chen D, Nikolovska-Coleska Z, Zou F, Yu J and Zhang L: Mcl-1 degradation is required for targeted therapeutics to eradicate colon cancer cells. *Cancer Res* 77: 2512-2521, 2017.
51. Hsu C, Lin LI, Cheng YC, Feng ZR, Shao YY, Cheng AL and Ou DL: Cyclin E1 inhibition can overcome sorafenib resistance in hepatocellular carcinoma cells through Mcl-1 suppression. *Clin Cancer Res* 22: 2555-2564, 2016.
52. Tutusaus A, Stefanovic M, Boix L, Cucarull B, Zamora A, Blasco L, de Frutos PG, Reig M, Fernandez-Checa JC, Marí M, *et al*: Antiapoptotic BCL-2 proteins determine sorafenib/regorafenib resistance and BH3-mimetic efficacy in hepatocellular carcinoma. *Oncotarget* 9: 16701-16717, 2018.
53. Hikita H, Takehara T, Shimizu S, Kodama T, Shigekawa M, Iwase K, Hosui A, Miyagi T, Tatsumi T, Ishida H, *et al*: The Bcl-xL inhibitor, ABT-737, efficiently induces apoptosis and suppresses growth of hepatoma cells in combination with sorafenib. *Hepatology* 52: 1310-1321, 2010.
54. Ivanisenko NV, Buchbinder JH, Espe J, Richter M, Bollmann M, Hillert LK, Ivanisenko VA and Lavrik IN: Delineating the role of c-FLIP/NEMO interaction in the CD95 network via rational design of molecular probes. *BMC Genomics* 20 (Suppl 3): S293, 2019.
55. Jiang Z and Clemens PR: Cellular caspase-8-like inhibitory protein (cFLIP) prevents inhibition of muscle cell differentiation induced by cancer cells. *FASEB J* 20: 2570-2572, 2006.
56. Guo ZL, Li JZ, Ma YY, Qian D, Zhong JY, Jin MM, Huang P, Che LY, Pan B, Wang Y, *et al*: Shikonin sensitizes A549 cells to TRAIL-induced apoptosis through the JNK, STAT3 and AKT pathways. *BMC Cell Biol* 19: 29, 2018.
57. Kim S, Woo SM, Min KJ, Seo SU, Lee TJ, Kubatka P, Kim DE and Kwon TK: WP1130 enhances TRAIL-induced apoptosis through USP9X-dependent miR-708-mediated downregulation of c-FLIP. *Cancers (Basel)* 11: 344, 2019.
58. Gu FM, Li QL, Gao Q, Jiang JH, Huang XY, Pan JF, Fan J and Zhou J: Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3. *World J Gastroenterol* 17: 3922-3932, 2011.
59. Tai WT, Cheng AL, Shiao CW, Huang HP, Huang JW, Chen PJ and Chen KF: Signal transducer and activator of transcription 3 is a major kinase-independent target of sorafenib in hepatocellular carcinoma. *J Hepatol* 55: 1041-1048, 2011.
60. Huang CY, Lin CS, Tai WT, Hsieh CY, Shiao CW, Cheng AL and Chen KF: Sorafenib enhances radiation-induced apoptosis in hepatocellular carcinoma by inhibiting STAT3. *Int J Radiat Oncol Biol Phys* 86: 456-462, 2013.
61. Chen KF, Tai WT, Liu TH, Huang HP, Lin YC, Shiao CW, Li PK, Chen PJ and Cheng AL: Sorafenib overcomes TRAIL resistance of hepatocellular carcinoma cells through the inhibition of STAT3. *Clin Cancer Res* 16: 5189-5199, 2010.
62. La Tessa C, Berger T, Kaderka R, Schardt D, Körner C, Ramm U, Licher J, Matsufuji N, Vallhagen Dahlgren C, Lomax T, *et al*: Out-of-field dose studies with an anthropomorphic phantom: Comparison of X-rays and particle therapy treatments. *Radiation Oncol* 105 1: 133-138, 2012.
63. Loeffler JS and Durante M: Charged particle therapy-optimization, challenges and future directions. *Nat Rev Clin Oncol* 10: 411-424, 2013.
64. Dolcet X, Llobet D, Pallares J and Matias-Guiu X: NF- $\kappa$ B in development and progression of human cancer. *Virchows Archiv* 446: 475-482, 2005.
65. Grivennikov SI and Karin M: Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev* 21: 11-19, 2010.
66. Yu H, Lee H, Herrmann A, Buettner R and Jove R: Revisiting STAT3 signalling in cancer: New and unexpected biological functions. *Nat Rev Cancer* 14: 736-746, 2014.

